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13. ABSTRACT (Maximum 200 Words)

NF1 is characterized by benign Schwann cell tumors called neurofibromas; complex forms can become malignant (MPNST). Little is known about involvement of steroid hormones in NF1 tumors. This work will examine the role of estrogen and progesterone in NF1 tumors, to test the hypothesis that human neurofibroma (and/or MPNST) Schwann cells have increased growth or decreased apoptosis related to steroid hormones. Specific Aim 1 is examining steroid hormone receptor expression in human normal, NF1 neurofibroma and MPNST Schwann cells. Real-time PCR shows very low levels of these receptor transcripts in untreated normal and tumor cells, with very little change in the tumors (less than 2-fold). There is no detectable estrogen receptor in normal Schwann cells or NF1 tumors by immunohistochemistry (IHC). However, IHC assay for progesterone receptor was weakly positive in 33% of neurofibromas. Specific Aim 2 is testing in vitro response of tumor cells to hormones. RT-PCR, proliferation assays, and apoptosis assays have thus far shown very few significant responses of the neurofibroma/MPNST Schwann cell cultures to hormones or antagonists. Specific Aim 3 tests in vivo hormone response of human tumor cells xenografted into Nf1 mouse nerve. Thus far, one MPNST has shown a dramatic effect with estrogen, with other cultures still under analysis but not showing such obvious results.

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INTRODUCTION

Clinical literature suggests that steroid hormones may play a role in neurofibromatosis 1 (NF1) since neurofibroma growth shows some parallels with hormonal changes (Dugoff and Sujansky, 1996). For example, many patients develop neurofibromas at puberty, pregnancy often increases tumor size/number, women with NF1 may have a higher risk of malignancy and a higher neurofibroma burden, and neurofibroma development often slows in older adults. The steroid hormone field is being intensely researched in many types of cancer, but virtually nothing is known about these pathways in normal or NF1-tumor derived Schwann cells. Schwann cells comprise the bulk of these tumors, in which a large percentage are somatically mutated and clonally expanded (Rutkowski et al., 2000; Wallace et al., 2000; Serra et al., 2000).

Our goal is to characterize the growth responses of human normal and NF1 tumor Schwann cells (neurofibromin-negative) to steroid hormones, focusing on estrogen and progesterone. The hypothesis is that human neurofibroma (and MPNST, malignant peripheral nerve sheath tumor) Schwann cells have increased hormone responsiveness compared to normal Schwann cells, leading to tumor growth. The role of the hormone receptors is a growing topic in cancer research, but little is known about these in normal or NF1 Schwann cells.

In Specific Aim 1, we will determine steroid hormone receptor expression in human normal, NF1 neurofibroma, and NF1 MPNST Schwann cells, pre- and post-hormone treatment by studying archived material and cell culture materials. We have a number of NF1 tumor-derived enriched Schwann cell cultures that have been found to lack neurofibromin and will be the basis for this work (Muir et al., 2001). In Specific Aim 2, we will test *in vitro* responses (proliferation and cell survival) of cultured human Schwann cells from normal nerve, NF1 neurofibromas and NF1 MPNSTs, to estrogen and progesterone and their antagonists. Specific Aim 3 will test *in vivo* responses (proliferation and survival) of NF1-derived tumor Schwann cell lines to estrogen and progesterone, through a xenoplant model in *Nf1 / scid* mice.

Regardless of the data that emerge, whether showing positive or negative or mixed response, this will be an important step in scientific knowledge about the role of these hormones in NF1. This will have impact on patients who are candidates for hormone therapies, and may lead to new targets for anti-tumor therapies.

Task 1. To characterize the steroid hormone receptor profile in 24 normal and NF1 tumor Schwann cell cultures pre- and post-hormone treatment (months 1-30).

Progress on Task 1a: "To measure initial expression, RNA from cells grown under normal culture conditions will undergo semi-quantitative RT-PCR to evaluate transcript levels of steroid receptors, a quantitation control, and selected downstream targets. Primary tumor samples corresponding to cultures will be surveyed as well. Immunohistochemistry of primary tumor sections will also be done with for receptors showing transcripts (months 1-12)."

Table 1 shows the data from samples that have been analyzed for estrogen receptor alpha (ERa), estrogen receptor beta (ERb), progestrone receptor (PR), and androgen receptor (AR) expression through use of much more sensitive method than originally planned, real-time polymerase chain reaction (RT-PCR, Roche LightCycler). The data are shown as a ratio relative to the averaged results from 2 normal Schwann cell cultures, all normalized with respect to the HPRT housekeeping gene. The studies were done in duplicate and averaged, to ensure consistency of data. All of these receptor levels were quite low (higher threshold cycle number to reach reliable quantitative detection (e.g. ER alpha threshold cycle number was typically 6-8 cycles more than HPRT). In Table 1, blanks indicate un-detectable levels, and ND indicates not determined. Current data include primary samples from 13 dermal neurofibromas and 8 dermal cultures, 7 primary plexiform neurofibromas and 8 plexiform cultures, 2 primary MPNST samples and 2 MPNST cultures; additional samples are being done during the last year). There were no dramatic changes in expression compared to normal, and these have been analyzed thus far with a Student's 2-tailed t test, as is standard in the literature. This compared the means between normal Schwann cell transcript levels with the levels in the tumor-based samples, after cycle number normalization to HPRT. Cultures and primary tumors were examined separately, within each category of dermal, plexiform, and MPNST. All fold changes were below 2-fold in either direction, and a few comparisons approached significance (see Table 2 below). Progesterone receptor (PR) tended to be the most elevated, but only one category's comparison closely approached significant (primary dermal tumors). Androgen receptor (AR) also tended to be slightly elevated in most primary tumors, less so in the cultures, and primary dermal tumors yielded the best statistic ($p = 0.076$). ER alpha (estrogen receptor alpha) had a trend in MPNST cultures ($p = 0.063$) although only two samples were in this category. Only one plexiform culture had detectable ER beta (estrogen receptor beta) levels, and that was significantly different from normal ($p=0.008$). This latter may be a legitimate finding since the other samples had even less ER beta message, although being un-measurable they were not in the statistical analysis done to this point. This would fit with our original hypothesis that ER beta has tumor suppressor function in Schwann cells, and that this is decreased in NF1 tumor Schwann cells. Final statistics will be calculated once all the data have been generated.

Table 2. T test analysis of real-time PCR data from Table 1 comparing normal Schwann cell values to tumor values (showing p values from calculations that used data points out to 4 decimal places)(bolded values are significant or approaching significance at $p \leq 0.05$).

Comparison	ER alpha	ER beta	PR	AR
Normal vs. dermal primary	0.29031	0.43668	**0.06470**	**0.07644**
Normal vs. dermal cultures	0.99994	0.58132	0.42185	0.68481
Normal vs. plexiform primary	0.19306	0.45854	0.15276	0.11315
Normal vs. plexiform cultures	0.45206	**0.00815**	0.63807	0.85773
Normal vs. MPNST primary	0.62039	0.76765	0.12136	0.26863
Normal vs. MPNST cultures	**0.06279**	0.92462	0.17654	0.48692

Immunohistochemistry was expanded and clearly showed no detectable levels of estrogen receptors at the level of this sensitivity. However, PR in 54 tumor sections (24 dermal, 25 plexiform, 5 MPNST) was weakly positive in 18, and strongly positive in 1 (a plexiform tumor from a non-pregnant female). This is very consistent with data published earlier in 2003 by McLaughlin and Jacks, which suggests that progesterone may play a role in some neurofibromas. Of interest, based on their paper and our real time PCR data of cultures vs. primary, it is possible that these receptors are on cells other than Schwann cells. We are currently staining several samples from neurofibromas received from a pregnant woman, to see if there is a higher level of PR given the circulating progesterone. None of the changes appear to correlate with gender of the sample, but we did not statistically analyze that yet since we are still generating data and the numbers in each category would be small. Based on our data, it is clear that protein-level approaches such as Western blot will not be sensitive enough to measure these receptors in neurofibromas or their Schwann cell cultures. Our conclusion at this point is that the steroid receptor ligands are differentially expressed in neurofibromas compared to normal Schwann cells, but that these differences are heterogeneous in some cases, and do not yet reach statistical significance with the exception of decrease in ER beta. There are trends that might be worth pursuing in the future, however.

Table 1: Light Cycler real-time PCR data.

Tumor Type	Samples	ER alpha	ER beta	PR	AR
Dermal cultures					
	cNF96.5g	1.0	-	1.1	1.1
	cNF97.2a	-	-	-	1.2
	cNF98.4a	0.9	1.0	1.1	1.1
	cNF98.4d	1.0	0.9	1.1	1.1
	cNF97.5	0.9	1.0	-	1.0
	cNF99.1	1.1	-	1.2	1.2
	UF 17T1c	1.0	-	-	0.9
	UF 17T2c	1.0	-	-	0.8
Plexiform cultures					
	pNF95.11b	0.8	1.0	1.0	1.1
	pNF97.9	1.0	-	1.2	1.1
	pNF98.3	1.0	-	0.9	0.9
	pNF99.5	-	-	-	0.8
	pNF00.6	1.0	-	1.0	0.9
	pNF00.8	1.0	-	1.1	1.0
	pNF02.6	1.0	-	1.0	1.1
	UF 550Tc	1.1	-	1.1	1.4
MPNST cultures					
	sNF96.2	-	-	-	1.2
	sNF94.3	0.7	0.9	1.2	1.3
Primary dermal tumors					
	UF 328T7	1.1	-	1.9	1.4
	UF 80T2	1.3	-	1.7	1.4
	UF 80T6	1.3	-	1.6	1.5
	UF 158T3	1.2	1.2	1.4	1.2
	UF 486T1	1.1	1.0	1.5	1.0
	UF 486T5	1.3	1.1	1.7	1.1
	UF 495T2	1.3	1.2	1.5	1.2
	UF 495T3	1.3	1.2	1.4	1.2
	UF 549T	-	-	1.6	1.4
	UF 743T1	1.2	1.2	1.4	1.2
	UF 743T2	1.3	1.2	1.5	1.2
	UF 743T4	1.2	1.2	1.4	1.1
Primary plexiform tumors					
	UF 362T	-	1.2	1.5	1.6
	UF 154T	1.3	1.1	1.6	1.3
	UF 429T	1.2	1.2	1.4	1.2
	UF 537T	-	-	1.3	1.0
	UF 714T	1.2	1.2	1.3	1.2
	UF 746T1	1.4	1.1	1.7	1.5
	UF 787T1	1.2	-	1.6	1.4
Primary MPNSTs					
	UF 459T	1.1	1.1	1.1	1.0
	UF 820T	1.0	1.1	1.3	1.1
Controls					
	MCF7	1.5	1.0	1.6	ND
	Testis	ND	ND	ND	1.2
	Caco2	ND	1.0	ND	ND

Progress on Task 1b: "Cultures treated in Task 2 will be analyzed with RT-PCR as above. (months 3-30)."

Real-time PCR for the steroid receptors and putative downstream gene targets (ER alpha and beta, PR, ERBB2 (the neu oncogene), EGFR (epidermal growth factor receptor), and VEGF (vascular endothelial growth factor)) is well underway for the tumor Schwann cell cultures treated with the hormones or SERMs. Currently, treatments have been done for Schwann cell cultures from 2 normals, 3 MPNSTs, 4 plexiform neurofibromas, and 3 dermal neurofibromas. RNA samples are being collected at day 0, 1, 3, and 7 after initiating drug treatment of the cultures. Not all have yet been analyzed with real-time PCR at all loci. ER beta has also been done but the data are not as finalized as in Table 3, in part because a number of the samples had no detectable ER beta. Of the samples analyzed so far, the levels of the receptors or downstream target genes are within the range 0.87 to 1.54 (most much closer to 1 than that), as shown in Table 3 below (a sampling of results showing the widest range of results). Thus, there are not fold-change level differences thus far. With each assay, a control of MCF7 breast cancer cells + estrogen (3 day) was a control that consistently showed slight decrease in ER alpha and increase in PR (one example shown in Table 3), consistent with the literature (e.g. de Cremoux et al., 2003). This verifies that the system is working, but also indicates that even small changes in expression of these genes are not unexpected yet could be associated with a biological effect. We have not yet done statistics since much of the real-time PCR data has yet to be analyzed, and there will be a number of statistical tests to do. However, since the range of differences is close to the range of values for no treatment (day 0 vs. 1 vs. 3 vs. 7, which should all be identical in theory), then it is possible that there may not be many significant changes. Based on our data and the literature, RNA-level analysis is difficult due to the scant amounts of message and relatively small changes in response to treatment. However any trends will give us clues about downstream pathway activity, and future work could analyze that at a protein or cell level.

Table 3. Real-time PCR results examining transcript levels of genes putatively downstream of steroid hormone pathways in Schwann cells. Data are shown as ratio of transcript at days under treatment (1,3,7) compared to day zero. Numbers above 1 represent a relative increase in that transcript, below 1 a decrease. ND = no detectable transcript. F is free (no treatment), E is estrogen treatment, and P is progesterone treatment.

Sample	EGFR	ER alpha	ERBB2	PR	VEGF
sNF94.3 – 0	1	ND		1	1
sNF94.3 – 1F	1.01	ND	1.001	1.01	0.98
sNF94.3 – 1E	1.02	ND	1.029	1	0.93
sNF94.3 – 1P	1.04	ND	1.03	0.99	0.95
sNF94.3 – 3F	1.05	ND	1.034	1.04	0.96
sNF94.3 – 3E	1.05	ND	1.05	1.05	0.96
sNF94.3 – 3P	1.05	ND	1.054	1.03	0.95
sNF94.3 – 7F	1.07	ND	1.075	1.05	0.93
sNF94.3 – 7E	1.03	ND	1.058	1.05	0.93
sNF94.3 – 7P	1.06	ND	1.071	1.06	0.93
pNF99.5 -- 0	1	1	1	1	1
pNF99.5 – 1F	0.97	0.87	0.983	1.26	0.98
pNF99.5 – 1E	1	0.87	0.983	ND	0.98
pNF99.5 – 1P	0.99	ND	0.987	ND	0.99
pNF99.5 – 3F	1.08	0.91	0.99	ND	0.98
pNF99.5 – 3E	1.01	0.88	0.988	ND	0.97
pNF99.5 – 3P	1.03	ND	0.978	ND	0.99
pNF99.5 – 7F	1.02	1.01	0.956	1.27	0.95
pNF99.5 – 7E	0.95	0.89	0.955	ND	0.94
pNF99.5 – 7P	0.98	ND	0.962	1.54	0.95
Normal SC - 0	1	1	1	1	1
Normal SC – 1F	1	0.98	0.995	0.95	0.93
Normal SC – 1E	0.99	0.97	0.991	0.95	0.91
Normal SC – 1P	0.98	0.98	1.005	0.96	0.92
Normal SC – 3F	1.08	0.97	0.958	0.97	0.89
Normal SC – 3E	1.07	1.04	0.988	1.03	0.92
Normal SC – 3P	1.05	1.01	0.952	1	0.9
Normal SC – 7F	1.07	1	0.963	1.06	0.93
Normal SC – 7E	1.08	1	0.957	1.05	0.94
Normal SC – 7P	1.07	0.99	0.945	1.05	0.92
cNF97.2a - 0	1	1	1	1	1
cNF97.2a – 1F	1	1.14	1.002	ND	0.92
cNF97.2a – 1E	0.96	0.93	0.988	ND	0.96
cNF97.2a – 1P	0.98	ND	1.002	0.96	0.94
cNF97.2a – 3F	1.02	1.15	0.99	1.03	0.99
cNF97.2a – 3E	1.02	0.96	0.976	0.99	0.96
cNF97.2a – 3P	1.01	1.14	0.989	0.95	0.95
cNF97.2a – 7F	1.02	1.11	1.003	1	0.97
cNF97.2a – 7E	1	0.93	1.007	0.99	0.92
cNF97.2a – 7P	1.01	1.11	0.995	0.98	0.96
MCF7 - 0	1	1	1	1	1
MCF7 –3E	0.87	0.93	0.932	1.03	1

Progress on Task 1c: "Cultures positive for receptors by RT-PCR (pre- and/or post-treatment) will be analyzed by Western blot and immunofluorescence (months 3-30)."

Based on the Task 1a and 1b results, Task 1c is no longer applicable because of the extremely low levels of the molecules, even in response to treatment. Reliable quantitative protein level analyses cannot be done.

Task 2. To measure cultures' proliferative and apoptotic response to hormone and SERM/antagonist treatment (months 1-30).

Progress on Task 2a: "Cultures will be treated with hormones/SERMs separately and proliferative response measured with BrdU assay, and cell survival measured by counting and TUNEL assay. (months 3-18)."

The proliferation and TUNEL assays have been perfected and have thus far been performed on most of the planned cultures. These cells were grown in steroid-hormone-depleted media, and subsequently subjected to the following independent treatments for 7 days: 100 nM estradiol, 100 nM progesterone, 1 micromolar ICI182,780 (estrogen SERM), 1 micromolar tamoxifen (another SERM), no ligand, and for cultures requiring neuregulin for proliferation, neuregulin alone was measured. Data are being collected in duplicate (assays done at a different time). More samples remain to be done. However, thus far there appears to be no consistent dramatic effect of the ligands on proliferation. Proliferation and apoptotic indices are shown below in Tables 4 and 5 for one set of data (additional and replicate assays being finished and analyzed now). These are based on percentage of the cells that were positive for each measurement (BrdU for proliferation; TUNEL staining for apoptotic cells).

Table 4. Proliferation Indices for Cultures. Treatments are 100 nM estradiol, 100 nM progesterone, 1 uM ICI182780, 1 uM mifepristone (RU486), 1 uM tamoxifen, and neuregulin (NRG, Schwann cell mitogen). N = normal Schwann cell culture, D = dermal neurofibroma Schwann cell culture, P = plexiform neurofibroma Schwann cell culture, M = MPNST culture. Bold numbers indicate statistically significant effect of the treatment compared to no ligand ($p \leq 0.05$, using a Z test with correction for testing equality of 2 proportions).

Sample ↓	estradiol	Progest.	ICI	RU486	tamoxifen	NRG	No ligand
N pn02.3	13.04	20.83	**3.52**	14.35	**7.51**	--	18.97
N pn97.4	17.52	15.69	13.53	**20.59**	**9.96**	**23.55**	15.35
N pn02.8	4.8	2.68	6.98	1.6	2.84	**12.12**	3.38
D cNF97.2a	**4.52**	7.12	**3.25**	7.53	5.67	**14.08**	9.26
D cNF99.1	12.06	15.44	**2.46**	**23.32**	15.69	--	15.89
D cNF97.5	**28.69**	**22.75**	**25.12**	**28.22**	17.56	**27.03**	16.85
D cNF97.2	2.28	2.12	1.15	2.05	1.79	**4.91**	2.02
P pNF99.5	1.06	1	0.46	0.92	1.08	**15.4**	1.41

P pNF01.1	15.43	15.45	**11.15**	16.42	12.26	12.73	15.56
P pNF02.6	7.25	5.26	6.74	1.44	5.85	2.38	3.73
P pNF00.13	**10.42**	9.7	9.7	8.11	**12.31**	4.94	6.34
M sNF96.2	72.99	**74.3**	65.77	71.8	72.87	--	68.82
M sNF02.2	44.41	**61.94**	**40.46**	48.71	**57.16**	**61.11**	49.85

Table 5. Apoptotic Indices for Cultures. Treatments are 100 nM estradiol, 100 nM progesterone, 1 uM ICI182780, 1 uM mifepristone (RU486), 1 uM tamoxifen, no serum, and no ligand. N = normal Schwann cell culture, D = dermal neurofibroma Schwann cell culture, P = plexiform neurofibroma Schwann cell culture, M = MPNST culture. Bold numbers indicate statistically significant ($p \leq 0.05$).

Sample	Estradiol	Progest.	ICI	RU486	tamoxifen	No serum	No ligand
N pn02.3	**1.02**	2.99	2.22	**1.28**	3.14	2.4	4.64
N pn97.4	1.71	1.29	1.18	2.14	2.24	**6.35**	1.44
D cNF97.2a	6.05	5.34	5.58	4.75	**11.02**	**17.36**	4.98
D cNF99.1	6.05	5.26	3.49	7.05	8.67	0.9	6.16
P pNF99.5	**5.66**	3.27	4.87	2.25	4.28	**11.61**	2.08
P pNF01.1	2.14	2.58	**0.89**	1.58	2.23	3.18	2.7
P pNF02.6	3.43	1.94	1.86	0.49	2.72	**5.11**	1.44
P pNF00.13	2.54	2.23	1.62	3.09	1.58	1.52	1.49
M sNF96.2	1.75	2.25	**3.52**	**3.76**	**3.23**	--	1.08
M sNF02.2	2.21	0.99	2.11	2.31	1.02	**7.12**	1.18

ICI 182,780 appears to induce a significant decrease in proliferation in five cultures, the most widespread trend, including in one normal culture (although one dermal culture had a significant increase in proliferation). No serum increased apoptosis as expected as a form of control, but there wer no significant effects on apoptosis from progesterone, and only 2 cultures each responded significantly to the other ligands. However, replicates have not yet been analyzed to see if the trends are reproducible for apoptosis or proliferation. Neuregulin induced proliferation in most cultures, which was a positive control for those known to respond to this growth factor. There were scattered other responses to several treatments, but no strong patterns. For example, 3 mifepristone treated cultures showed significant increases in proliferation, but no other cultures had a significant response. There are mixed results for tamoxifen. As expected, the cultures are responding heterogeneously relative to each other, within or between categories, which we believe in part reflects underlying differences between patients/tumors. Also, many of these effects are rather small, and thus the outcomes may be sensitive to subtle differences in assay conditions, despite attempting to keep as many variables as constant as possible.

Progress on Task 2b: "Cultures showing a response will undergo dose response testing, and assays in (a) will be repeated using combinations of appropriate hormones/SERMs/antagonists. (months 12-30)."

Dr. Campbell-Thompson, our co-investigator with substantial experience in steroid hormone work, indicates that dose response and combinatorial testing is not yet indicated because we've had no significant responses to date with relatively high levels of the drugs. Should any of the future samples show significant proliferation or apoptosis changes due to the ligands, however, we will expand into dose-response analysis for those.

Progress on Task 2c: "Statistical analysis (months 24-36)."

This is underway with current data, but won't be finalized until the end of the grant. We are working with a biostatistician from the UF Shands Cancer Center to ensure use of the best statistical tests for the various types of data being generated.

Task 3. To assay for *in vivo* proliferative or survival response of tumor cells to estrogen and progesterone. (months 12-36).

Progress on Task 3a: "Select 6 tumor and 2 normal Schwann cell cultures (in part based on *in vitro* results)."

Two MPNST cultures were chosen, and since one showed an interesting phenotype which might be related to gender issues, a third one is being tested as well. Four plexiform cultures have been selected, and one dermal culture thus far. More will be chosen during the coming year.

Progress on Task 3b: "For each culture, sterilize 15 *scid* mice heterozygous for an *Nf1* mutation, inject cells, and treat mice with estrogen, progesterone, or placebo."

The colony of Nf1/scid mice suffered a decrease in fecundity this year, and thus fewer animals were available for surgery. This was apparently due to inbreeding effects and the fact that scid mice have shortened lifespans (most die by 12-14 months). Thus, many of our breeding females were only producing a total 3 litters, with an average of 3.5 surviving pups each litter. Surgeries require heterozygous females, which are expected to be 25-33% of any litter depending on the mating, and thus we had a scant number produced even with 10-20 mating cages. We expected a much greater output of offspring this year. Thus, we purchased 20 new scid females from Jackson Labs, and expanded the size of the breeding colony to accommodate as much breeding as possible. Within the last couple of months, the production of pups has increased greatly. However, because of this unexpected turn of events, and unplanned increases in mouse housing expenses at UF, our mouse costs were 4-times as much as budgeted for this year. Due to the delay/reduced animal numbers and the increased costs, we have been forced to change our priorities since we may not be able to perform as many in vivo experiments as

planned. Thus, we are currently aiming for n=3 mice for each culture for each condition (a total of 9 rather than 15). Since both sciatic nerves are being injected, this is a total of 6 nerves per treatment per culture, which is sufficient for most statistics. In addition, we have observed very consistent growth patterns within each treatment/culture, and thus feel that 6 nerves rather than 10 should adequately answer questions about effects of the hormones. Should we find the time/money to increase our numbers beyond 3 mice per treatment, we will do so. An additional confounding factor has been that a few of the benign Schwann cell cultures have not been able to proliferate for very many passages, and obtaining enough cells to inject 15 mice is not feasible (5×10^5 cells are used per nerve). These cultures are in limited supply. Thus far, we have injected the 9 mice for two MPNST cultures. Seven mice have been done for another MPNST. Of two plexiform cultures done so far, one has had 6 mice done, and other 7. One dermal culture has also had 9 mice injected. We have had a total of 4 mice die at some point after surgery, prior to the 2-month harvest (between 1 and 2 months; these deaths were mostly accidental and not at all related to the experiment). 60-day release pellets are used and thus the protocol calls for harvesting the nerves at day 60. We still harvested the nerves of the mice that died prematurely, for observational data, but will try to do more mice to finish the data for the full growth period to add to the statistical analysis. We will not be doing the normal Schwann cell injections since Dr. Muir's lab has now done this several times with intact female mice and has consistently seen no growth of several normal human Schwann cell cultures. Thus, that baseline measure has already been established.

Table 6 below shows the data thus far analyzed for tumor size, which utilizes the IMAGE ProPlus computer system to calculate number of pixels in area of tumor, from 3 slides of each in same relative location through the nerve for each sample. There are still more nerves to be counted and analyzed, so these are preliminary data. There are some experiments remaining in this in vivo portion of the project, to be followed by completion of microscopic data analysis and substantial statistical analysis to derive all the conclusions possible.

Table 6. In vivo tumor graft data, average area of tumor in pixels (5X magnification). Only the sNF96.2 tumor had a statistically significant change in tumor size due to a treatment (estrogen) compared to placebo.

	SNF96.2	SNF94.3	CNF97.5	PNF95.11b	PNF00.13
placebo	671,165	315,639	312,116	265,548	233,262
estrogen	1,354,833*	339,401	316,326	238,056	205,399
progesterone	798,163	333,066	270,234	240,671	214,182

Progress on Task 3c: "Perform proliferation assay (Ki67 staining) and TUNEL assay on xenografts by analysis of tissue section, and statistical analysis."

These assays have now been perfected. Serial sections are made of the harvested mouse nerves, and a template is used to choose several sections each at specified intervals, for GST staining (identifies human cells), Ki67 staining for proliferation, and TUNEL for apoptosis. Several measures are taken—area of tumor at maximum diameter, % of human cells that are Ki67-positive, and % of human cells that are TUNEL-positive. Most of the 44 mice treated in 3b have had their nerves harvested and are being

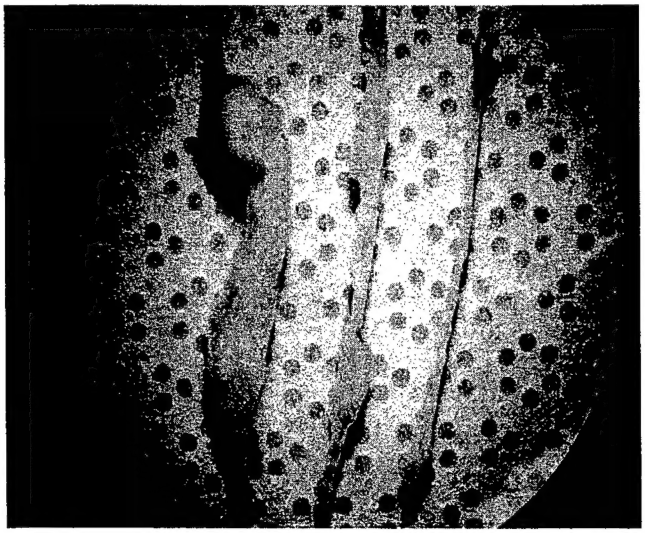
analyzed. Thus far, one MPNST (sNF96.2, from a male) has reproducibly shown dramatic growth in vivo under the influence of estradiol, with less of an effect with progesterone (see Fig. 1 and 2 and Table 7 below). The tumor cells grew virtually the entire length of the sciatic nerve compartment, including back up to the spinal nerve root. The other MPNST (sNF94.3, from a female), which grows more aggressively in vitro (so fast that it could not be subjected to the proliferation assay), did not show any substantial effect from any hormones. Because of this, we chose to analyze at least one additional MPNST, which is underway, to see if any patterns emerge in MPNSTs with regard to gender of donor/recipient, or which hormone (if any) shows an effect. This sNF96.2 culture did not show significant alterations of proliferation or apoptosis in the in vitro analysis, suggesting that the in vivo situation is an important additional assay for measuring hormone effects. All of the cultures injected have shown viability of the human cells in the nerve compartment, and thus we are obtaining a good tumor model.

As a preliminary analysis, Table 7 shows the relative proliferation/apoptosis ratio for each treatment, normalized to placebo for the data we have at the moment. We had planned to use GST staining to quantify human cells for a proliferative or apoptotic index, but the GST counting is not accurate enough to be reliable for calculations (only the cytoplasm stains, and it can be difficult to see exactly how many positive cells are there). Thus, we are deriving data about the relative effect of the ligands on proliferation and apoptosis by calculating their ratio for each sample/treatment (# of Ki67 positive cells in the entire tumor area/ #TUNEL positive cells in entire tumor are) and normalizing to the placebo ratio. Thus, a final ratio of 1 indicates similar proliferation and apoptosis in the tumor; less than 1 indicates relatively more apoptosis occurred, and greater than 1 indicates that more proliferation was present. As can be deduced from the data, some of the ligands had substantial effects, but not in the same direction. The two plexiform cell cultures had virtually no proliferation at all, but estrogen increased the relative apoptosisin pNF00.13. Estrogen had a positive proliferative effect in one of the MPNSTs and the dermal culture. Thus, we are seeing some reproducible effects, but they are heterogeneous between patients, tumor types, ligand, and type of effect.

Table 7. Proliferation/apoptosis ratio normalized to placebo.

	SNF96.2	SNF94.3	CNF97.5	PNF95.11b	PNF00.13
estrogen	1.75	1.4	2.86	0	1.39
progesterone	0.5	2.5	0.28	0.56	0.86

Figure 1. Xenografted nerves with cell line sNF 96.2 under placebo (left bar) and 17β-estradiol (right bar) releasing pellets were sectioned and stained with polyclonal anti-human Ki-67 antibody. Immunoreactive cells from 3 animals with each treatment (2 nerves each mouse, 3 sections for each nerve and 3 sampled square units per section were counted (vertical axis is proliferative squares per square unit). Estradiol treatment produced a statistically significant increase in proliferation compared to placebo, the only culture yet analyzed that shows such effect.



A B C

Figure 2. sNF96.2 gross results. A and B represent nerves taken from xenografted mice treated with estradiol or placebo, respectively. C is a nerve from a PBS injected mouse for control. Dramatic growth of the tumor cells is evident with estradiol. Progesterone results were similar to placebo (data now shown).

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed that the basal steroid receptor levels in normal and NF1 neurofibroma Schwann cells are very low (supported by real-time PCR and immunohistochemistry data), with a large number of samples.
- Showed that progesterone receptor tended to be increased (less than 2-fold) in many primary tumors, and some cultured Schwann cells. Androgen receptor also tended to be increased, to a lesser degree.
- Completed proliferation and apoptosis assays for over half of the in vitro samples.
- Progressed on in vivo studies, with discovery of one culture showing dramatic response to estradiol in vivo.

REPORTABLE OUTCOMES

Two presentations at the NNFF International Consortium on the Molecular Biology of NF1 and NF2, June 2003 in Aspen, were related to this work. The first abstract referenced below (presented as a poster) is the most relevant for the steroid work; the second abstract relates to the xenograft system using the mouse colony developed for our work, and was an oral presentation by Dr. Perrin.

Fishbein L, Zhang X, Perrin G, Campbell-Thompson M, Muir D, Wallace M. Effects of steroid hormones in NF1 tumorigenesis.

Perrin G, Wallace MR, Muir D. Characterization of a reproducible xenograft model for NF1 plexiform neurofibroma.

CONCLUSIONS:

Analyses of primary and in vitro data suggest that steroid hormone receptor profiles in normal and NF1 tumor-derived Schwann cells are low and do not differ in a multiple-fold fashion, even in response to in vitro hormone treatment. There is heterogeneity in these data, as originally predicted. Although the data production and statistical analysis are still underway, early results indicate very few widespread differences in vitro, although several cultures appear to have specific significant responses to different ligands. The in vivo data suggest different effects of the ligands on different tumor cultures, again representing heterogeneity. Our early data also show a difference between in vitro and in vivo results, which is not unexpected, and was the basis for doing both approaches. The combination of these two methods will help address the question of whether there are detectable changes at the variables we are measuring, to help determine if steroid hormones might be having a biologically significant effect on neurofibroma Schwann cells, or if we can decipher categories of response that we can apply to these and other tumors.

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APPENDICES: None